Supplementary material

Supplementary Methods

Adenovirus production

The Fc domain, containing mutations in the IgG-R and complement binding domains, was amplified by PCR from an IL-10-Fc fusion plasmid (generously provided by Dr Terry Storm, Harvard University, USA). The extracellular domain of mouse N-cadherin was cloned with the Fc domain into pDC515 (Microbix). The secretion signal was cloned from full length N-cadherin onto the Fc fragment and cloned into pDC515. Both were recombined with the adenovirus genomic plasmid by co-transfection into 293 cells. The resultant adenoviruses were termed RAd SNC-Fc and RAd Fc (control). This chimeric molecule comprised of SNC and an antibody constant domain, enables protein A binding, as well as extending plasma half-life[1-3] and possibly increasing receptor–ligand interaction. Such immunoadhesins have use *in vivo* as potential therapeutic agents[4].

Purification and culture of mouse blood monocytes

Mouse peripheral blood monocytes were purified by Ficoll-Hypaque gradient (Ficoll-Paque Plus: Amersham Biosciences), followed by differential adherence and culture in 20ng/ml M-CSF for 7-10 days to induce differentiation into macrophages.

Purification of rabbit foam cell-macrophages

New Zealand White rabbits (Harlan, UK) fed a 1% cholesterol-supplemented diet had sterile sponges placed under the dorsal skin to generate foam cell macrophages, as described[5].

siRNA Knockdown

Two HP validated silencing RNA oligonucleotides (siRNA) for FGF-R1 and control (Allstars Negative control siRNA) were purchased from Qiagen (catalogue numbers SI02224677, SI02224684 and 1027281). VSMCs ($8x10^5$) were subjected to Amaxa nucleofection with

250pmol of FGF-R1 or control siRNAs using the VSMC kit and U-25 program following the manufacturer's instructions (Amaxa, Inc., Cologne Germany). Treated cells were analysed 24 hours after nucleofection. Knockdown of FGF-R was estimated as 85±7% (n=3, $p<0.05$) by quantitative PCR and was confirmed by immunocytochemistry (data not shown).

Western blotting

SDS lysed cell extracts were subjected to Western blotting as described previously[6]. Blots were incubated overnight at 4°C with primary antibodies diluted in Starting block (Pierce, Chester, UK). Antibodies were used at the following concentrations: total and pAkt (Cell Signalling, 1:1000). Bound antibodies were detected by rabbit anti-mouse horseradish peroxidase conjugated antibodies (Dako, High Wycombe, UK) and enhanced chemiluminescence (Amersham International, Little Chalfont, UK).

Galactolight assay

TOPgal transgenic mouse VSMCs were grown from aortic explants, as described previously[7]. These VSMCs contain the β-galactosidase gene under the control of the β-catenin promoter. TOPgal VSMCs were treated with 0.01% (w/v) trypsin, 1 mM $CaCl_2$, in PBS) and seeded into a 24 well plate in the presence of SNC-Fc or Fc and Fas-L. β-galactosidase activity was quantified using a chemiluminescent assay called Galactolight as described by the manufacturer's instructions (Tropix).

Aggregation and adhesion assays

Cell aggregation and cell-cell adhesion were determined as previously[8, 9].

Invasion of monocytes and proliferation of macrophages

Mouse peripheral blood monocyte invasion and macrophage proliferation were assessed as previously[10].

Quantitative PCR

Total RNA was isolated by the RNAeasy kit (Qiagen) was reverse transcribed and subjected to quantitative PCR for N-cadherin and FGF-R using Quantitect primers (Qiagen, QT00102837and QT00198548, respectively) as described by the manufacturer.

In vivo experiments

Quantification of plasma SNC-Fc and lipoprotein levels

Plasma samples were taken at 2, 6, 8, 14 and 28 days after RAd administration and levels of SNC-Fc were analysed by ELISA as described previously[6]. Plasma lipid profiles were analyzed in terminal plasma samples as previously described[11].

Immunohistochemistry

VSMCs, macrophages, and proliferating and apoptotic cells were identified by immunohistochemistry for α-smooth muscle cell actin, Mac-2, proliferating cell nuclear antigen (PCNA) and CC-3 as described previously[10]. Fluorescent immunohistochemistry for pAkt was performed using rabbit anti-pAkt antibody (Cell Signalling) diluted 1:25. Fluorescent dual immunohistochemistry for Mac-2 and N-cadherin or FGF-R1 was performed on control atherosclerotic plaques using 5 μg/ml rat anti-Mac-2 (Cedar Lane) and 2 μg/ml rabbit anti-N-cadherin (Santa Cruz) or rabbit anti-FGF-R1 (Cell Signalling) diluted 1:12.5.

In situ end labelling and actin dual immunohistochemistry

Apoptotic cells were identified by *in situ* end labelling (ISEL), performed as previously described[12]. This was followed by the smooth muscle α-actin immunohistochemisty protocol outlined above.

Identification of buried fibrous caps

Serial sections stained for elastin and α-smooth muscle cell actin were examined for the presence of structures rich in elastin and VSMCs and these were identified as buried fibrous caps, a surrogate marker of previous plaque instability, as previously described[13].

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Supplementary Figure Legends

Supplementary Figure I: The pro-survival effect of SNC requires the HAV motif.

Percentage of apoptotic VSMC (CC-3 ICC) 24 hours after FasL treatment with peptide (n=3).*significant difference from Fc control, $^{\$}$significant difference from HAV.

Supplementary Figure II: Expression of N-cadherin and FGF-R1 in monocytes and macrophages.

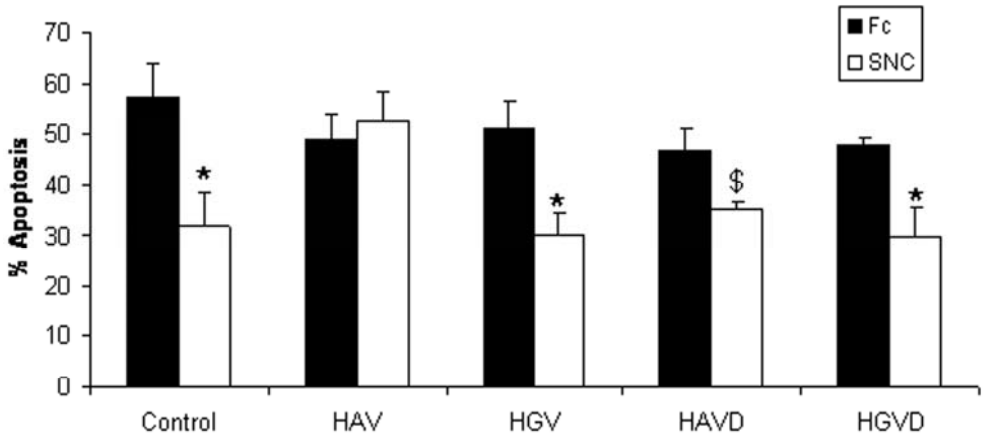
Table shows quantitative PCR results for N-cadherin and FGF-R1 in monocytes, macrophages and VSMCs expressed as copy number of mRNA.

Images show representative dual immunohistochemistry for N-cadherin (A, D) or FGF-R1 (B, E) in green and macrophages in red in PBS control atherosclerotic plaque. Arrowheads indicate macrophages expressing N-cadherin or FGF-R. Scale bar in panel A represents 50 μm and applies to panels A-C. Scale bar in panel D represents 15 μm and applies to panels D-F. Non-immune IgG is shown as negative control (C, F). Nuclei are stained blue with DAPI.

Supplementary Figure III: Schematic diagram of the mechanism of action of SNC

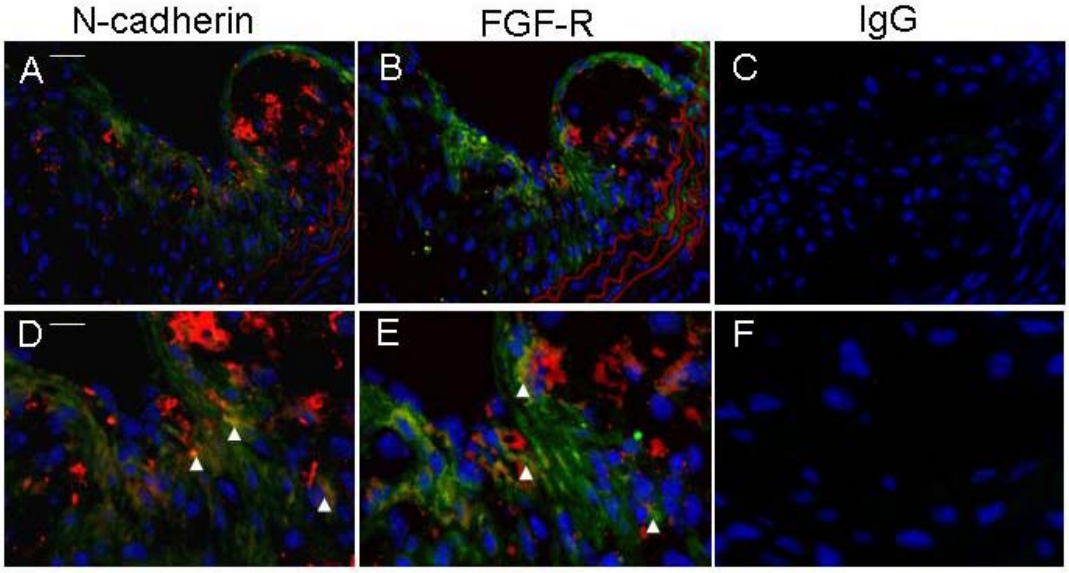
SNC may interact with full length N-cadherin which in turn binds to FGF-R or it may bind directly with FGF-R and full length N-cadherin. These interactions activate PI3-kinase and thereby activate Akt. Active Akt phosphorylates Bad which inhibits the interaction with Bcl-2, released Bcl-2 then provides a survival signal for VSMCs, inhibiting apoptosis.

Supplementary Figure I



Supplementary Figure II

Copy number of mRNA	N-cadherin	FGF-R1
Monocytes	13.7±13.7 (n=4)	4.2±2.3 (n=3)
Macrophages	1104±449 (n=4)	6.0±4.5 (n=3)
VSMCs	1707±871 (n=6)	168±76 (n=3)



Supplementary Figure III